PATENT
Attorney Docket No.0660-0135-OX CIP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
)
Shirley LONGACRE-ANDRE et al)
)
Serial No.:09/134,333)
)
Filed: August 14, 1998)
)
)
For: RECOMBINANT PROTEIN)
CONTAINING A C-TERMINAL)
FRAGMENT OF PLASMODIUM)
MSP-1)

Group Art Unit: 1641

Examiner: J.L. GRUN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF SHIRLEY LONGACRE

I, Shirley Longacre (formerly Shirley Longacre-Andre) do hereby declare the following:

1. I am one of the inventors of the present application. I have over 25 years of experience working on molecular biological approaches to parasitology and immunology. Since 1990, my research at the Pasteur Institute has been devoted to the production of recombinant analogs of merozoite surface proteins (MSPs) from three *Plasmodium* species infectious to humans, in the baculovirus / insect cell expression system. Until recently much of our work has focused on C-terminal MSP1 (p19) antigens, including the analysis of their structure and biological

properties, as well as their role in the induction of protective efficacy against malaria in primate systems. Indeed, this work provided the basis for the first determination of MSP1p19 crystal structure. Attached hereto as Annex I is my Curriculum Vitae, with a list of my publications relating to the present invention.

2. I have focused on MSP antigens, because the merozoite is the extracellular infectious form of the malaria parasite for erythrocytes, and its surface proteins are obvious vaccine targets since they are exposed to circulating antibodies and are presumed to play essential roles in the erythrocyte invasion process. A homologue of the major *P. falciparum* merozoite surface protein 1 (PfMSP1) is found on the merozoite surface in all species of *Plasmodium* studied to date, where it is anchored at its C-terminus by a glyco-lipid glycosylphosphatidyl-inositol (GPI) moiety. The *P. falciparum* MSP1 precursor is processed in at least two proteolytic steps, leading first to a noncovalently associated complex of MSP1 derived peptides, including a membrane bound 42 kDa C-terminal moiety. In a secondary processing step, the 42 kDa fragment is further cleaved to the C-terminal MSP1p19 GPI anchored protein with 96 amino acids, which remains on the merozoite surface during and after invasion. This small polypeptide has 12 cysteines and folds into two intimately associated epidermal growth factor (EGF)-like domains, each with three disulfide bridges. Thus, the MSP1p19 antigen has a very complex native conformational structure, which would have to be accurately and quantitatively reproduced in recombinant analogs to

induce a protective immunological response against malaria parasites. Another very important property of MSP1p19, rarely observed in parasite surface proteins, is that it displays very limited polymorphism in wild isolates of parasites from diverse geographical locations. Thus, a vaccine based on this antigen should protect very well against most, if not all, heterologous parasite strains found in any endemic region.

3. The above-captioned patent application relates to purified baculovirus recombinant MSP1p19 analogs from three different *Plasmodium* species infectious to humans: *P. falciparum, P. vivax* and *P. cynomolgi*. These constructs contain only sequences derived from MSP1 proteins, except for a C-terminal hexa-histidine tag used for metallo-affinity purification, and 2 residues created by the 6-base EcoRI restriction site joining nucleotide sequences coding for N-terminal (including signal sequences) and C-terminal portions of MSP1. These constructs are correctly processed by the baculovirus system to mediate protein secretion. Furthermore, the fact that the purified baculovirus recombinant MSP1p19 proteins could be crystallized, allowing the resolution of their 3-dimensional structure, shows definitively that these preparations are highly homogenous, with quantitative reproduction of the complex conformational MSP1p19 epitopes.

4. I have read the U.S. Official Action of December 19, 2005, as well as the claims, which are currently of record in the present application.

As I understand it, the Examiner has rejected the present application in view of several documents. I have read and I am familiar with the documents that were cited in the rejections. I have also read the response to this Official Action and agree with the interpretation of the two cited Longacre (1995) and Longacre et al (1994) references. More specifically, neither reference discloses the inhibition of parasitemia *in vivo* in a host infected with a *Plasmodium* parasite. Furthermore, neither reference refers to the use of alum or any other adjuvant for use in a vaccinating composition. It should be appreciated that without experimentally demonstrating that parasitemia can be reduced or inhibited *in vivo* in a host infected with a *Plasmodium* parasite infectious to humans, it could not be predicted at that epoch whether these sequences (Longacre 1995) or recombinant proteins (Longacre et al 1994) could in fact be useful in a vaccinating composition. Indeed, many types of MSP1 based recombinant proteins from *Plasmodium* species infectious to man have been described, (including the individual *E. coli* produced PfMSP1p19 EGF domains of Chappel and Holder), which never demonstrated protective efficacy when tested in primate challenge trials.

5. With respect to the other cited prior art of Holder and Chappel, i.e., U.S. Patent 5,720,959, to Holder et al and the article of Chappel and Holder (1993), it should be appreciated that these documents are from the same scientific group and relate to *E. coli* recombinant proteins corresponding to the two individual EGF-like domains from PfMSP1p19. I am familiar with the research done in the Holder group and have the following

comments to make with respect to U.S. Patent 5,720,959 and the paper of Chappel and Holder. U.S. patent 5,720,959 focuses primarily on polypeptides with the sequences described in Figs 1 or 2, which correspond to the two individual EGF-like domains produced as separate entities. It is further specified in this patent at column 2, lines 41 to 46, that no other naturally adjacent amino acid sequence be in the construct and thus only individual EGF-like domains are needed for antigenicity. U.S. Patent 5,720,959 discloses that recombinant proteins of the EGF-like domains are produced in *E.coli* and are indistinguishable from the native protein (at column 2, lines 33 to 39). However, it is a well-known observation that proteins expressed as intracellular proteins in *E. coli* do not form correct disulphide bonds, even when expressed as fusion proteins. Indeed, the only experiment to confirm correct conformation of the *E. coli* recombinant EGF-like domains, is a Western blot (Fig. 2 in Chappel and Holder, 1993) using a panel of 6 monoclonal antibodies, 5 of which bind to disulphide-constrained, reduction sensitive epitopes, and 3 of which were known to inhibit merozoite invasion *in vitro* (not *in vivo*). However, binding of antibodies to proteins on Western blots, is only a very preliminary, highly inaccurate technique to assess whether conformational structure is maintained quantitatively in all molecules of a purified recombinant protein preparation. Furthermore, U.S. Patent 5,720,959 discloses that the recombinant protein produced in *E. coli* and tested in mice is derived from *P. yoelii* MSP1 and contains both EGF-like domains in a single polypeptide, as in the native antigen. *P. yoelii* is a rodent malaria parasite and is not infectious in man. Only four species from the genus *Plasmodium* habitually infect man: *P. vivax, P. ovale, P.*

malariæ and *P. falciparum*, (although *P. cynomolgi*, which is highly homologous to *P. vivax*, is also known to infect humans). The *P. falciparum* recombinant MSP1 proteins described in the Holder patent have never been shown to have protective efficacy in a monkey model, regardless of the formulation used. The consequence of this will be discussed below under paragraph 8 below. Finally, although U.S. Patent 5,720,959 mentions the fact that alum can be used as an adjuvant, it was never tested with the recombinant construct in any of the examples; only Freund's complete and incomplete adjuvant (FCA/FIA) were used in the mouse model. It is well known in the immunological art that the use of different adjuvants can profoundly affect the strength and efficacy of the immune response to any given protein antigen, and this premise applies equally to recombinant *Plasmodium* proteins. Adjuvants are used primarily for soliciting and augmenting specified immune effector functions. In the case of the present patent application, adjuvants were required to induce significant antibody titers specific to the recombinant MSP1p19 immunogens. It was known at the time of the filing of the present patent application that the efficacy of recombinant MSP1 based vaccines was highly adjuvant dependent, such that different formulations of the same immunogen could have very different protective outcomes. Therefore, it was in no way predictable that alum would in fact work with the recombinant polypeptide(s) described either in the present patent application or in the U.S. Patent 5,720,959 without first being tested. Indeed alum is known to be a considerably less potent and efficient adjuvant than FCA/FIA. To my knowledge, the present patent application contains the only known demonstration(s) (shown both with the natural *P.*

cynomolgi / Macaca sinica and the experimental *P. falciparum / Saimiri scieurus* parasite/primate systems), that alum formulations of any recombinant C-terminal MSP1 antigen can be used to confer protection against malaria in primates. As evidence of this last statement, attached as Annex II, is the publication of Berghaus et al. (1996), *Infection and Immunity* 64, 3614-3629, concerning a recombinant C-terminal MSP1p19 of *Plasmodium falciparum* expressed in *E.coli* as a fusion protein with glutathione S-transferase (described in Berghaus and Holder (1994), *Molecular and Biochemical Parasitology* 64, 165-169). This construct is very similar to that described in U.S. Patent 5,720,959, and in Chappel and Holder in paragraph 6 below, except that the EGF1- and EGF2-like domains were expressed together in the same polypeptide, rather than individually. When this recombinant protein was administered to *Aotus nancymai* monkeys, either absorbed to alum or incorporated into liposomes, there was no protection against a challenge infection of *P. falciparum* infected erythrocytes. All of the monkeys became parasitemic and required treatment. Furthermore, Miller et al. (1997) remark regarding the development of malaria blood stage vaccines that "...to date, protective immunogens appear to require complete Freund's adjuvant (CFA); other adjuvants suitable for human use such as alum have not been effective."

6. With respect to the reference of Chappel and Holder, cited in the Official Action, there is a similar disclosure as that of the above patent to Holder et al. discussed in paragraph 5. However, data presented in this publication strongly support the contention that there are important

differences between the constructs of the present invention and those described in this reference. I would like to draw the attention of the Examiner to Figure 2 of this reference. This figure shows the reactivity of monoclonal antibodies such as those named 12. 8, 12.10 and 5B1, which are considered to be "protective" (i.e. shown to inhibit the invasion of red blood cells by merozoites *in vitro*), and a rabbit polyclonal antibody raised against affinity purified native MSP1, on immunoblots with "an empirically determined amount" of an insect cell product, S42ΔA, which contains 271 amino acids of the Wellcome strain MSP1 (residues 1433 to 1723) including both EGF-like domains fused to the amino terminal 34 amino acids of MSP1 to provide a signal for secretion (lane 1), and four *E. coli* GST-fusion proteins containing individual MSP1 EGF-like domains. In this figure the upper left hand image shows one gel stained with Coomassie Brilliant Blue (CBB) to indicate the amounts of protein loaded and the other gels were transfered to nitrocellulose. On the CBB stained gel the insect derived protein is not even readily visible, while the bacterial products are clearly present in quite large amounts, suggesting that there must be at least 10 to 100 fold more product. Nevertheless, the antibody reactivity with the insect protein is consistently as good or better than any of the bacterially derived products. This is observed most notably with the invasion inhibitory "protective" monoclonal antibodies and the polyclonal antisera raised to native MSP1. These results demonstrate that at best, only relatively small proportions, if any, of the bacterial products possess the native conformation, compared to the insect product.

7. To the best of my knowledge, there is no published or unpublished demonstration to date, 13 years after the PCT of U.S. patent 5,720,959 was filed, that recombinant polypeptides corresponding to individual MSP1 C-terminal EGF-like domains, produced in *E. coli* or any other expression system, can induce protective immunity against any *Plasmodium* parasite, in any host, using any adjuvant.

8. Furthermore, it should be noted that no other C-terminal MSP1 constructs other than those described in the present invention, have shown as complete and consistent protection in primate vaccination trials. Protective efficacy of the constructs of the present invention have been demonstrated preferentially using a parasite/primate test system with macaque monkeys (e.g. toque and rhesus monkeys), which are considered to be more predicative of human immune responses than new world monkeys such as *Aotus* and *Saimiri*, which are not naturally infected with *Plasmodium*. In contrast *Macaca sinica* (the toque monkey) is infected by *P. cynomolgi* in natural settings. While this system is more analogous to *P. vivax* infection in man, it could also be considered more predictive of efficacy against *P. falciparum* infection than the more artificial models involving *P. falciparum* itself. Six separate experiments carried out in the *Macaca sinica/P. cynomolgi* system using FCA/FIA, or Montanide ISA51 (similar to FIA, but human compatible) formulations of MSP1p19 of the present invention, with different batches of antigen or administered by the subcutaneous or intramuscular routes of injection, has resulted in 100% sterile, or nearly sterile immunity with barely detectable parasitemia on only 1 or 2 days after challenge. Furthermore in one

experiment performed using a baculovirus *P. falciparum* MSP1p19 construct in *Saimiri* monkeys, there was significant protection using alum as the adjuvant. It should be noted that all of the antibodies induced by the MSP1p19 antigens in these experiments, recognize exclusively non-linear reduction sensitive conformational epitopes, indicating that proper folding of the immunogens is essential for their protective efficacy.

9. Moreover in the immunization system described in paragraph 8, durable protection was conferred as measured by homologous or heterologous rechallenge after 4 to 6 months. This prolonged immunity could be due to either to a long-lasting effect of the immunogen itself or to effective boosting by very low or undetectable parasitemia. Heterologous rechallenge was undertaken since homologous rechallenge is a somewhat artificial situation due to the fact that in nature the extreme polymorphism of *Plasmodium* ensures that infections are rarely, if ever, mediated by identical strains as in homologous challenge. We have also further demonstrated excellent protection against heterologous rechallenge using MSP1p19 of the present invention and alum. To my knowledge no other malaria vaccine candidate, including other recombinant versions of MSP1p19 has been shown to confer <u>any protection against heterologous challenge or rechallenge</u>, much less the significant levels I have observed.

10. Finally it should be noted that since the FCA/FIA adjuvant is too toxic for human use, the demonstration in two experiments of significant partial protection of the MSP1p19 antigen formulated in alum, as well as

increased protection after heterologous rechallenge is of crucial importance since alum is the only adjuvant routinely licensed for human use. Moreover no adverse side effects were observed in any of the 90 primates that were vaccinated with the various baculovirus MSP1p19 antigens of the present invention.

11. In conclusion it should be said that none of the cited documents in the Official Action provide a vaccinating composition against a *Plasmodium* parasite that is infectious in man, which has been shown to induce an immune response that can inhibit parasitemia *in vivo* in a host infected with such a *Plasmodium* parasite.

12. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

16 June 2006

Date

Shirley Longacre

Annex I

Dr. Shirley LONGACRE, Ph.D.

Curriculum Vitae

Born 2 May 1946, Washington, D.C., U.S.A.
American and French nationality

Address : Laboratory of Parasite Vaccinology
Department of Parasitology and Mycology
Pasteur Institute, Paris

University degrees

Bachelor of Arts in Biology, Rice University, Houston, Texas, U.S.A. (1968)
Doctor of Philosophy in Biochemistry, University of California, San Francisco, U.S.A. (1976)

Research positions

1973-1975 : Teaching assistant, University of California, San Francisco
1976-1979 : Research assistant, University of Geneva (Switzerland)
1980-1981 : Research Associate, 2nd class, at the French National Center for Scientific Research (CNRS)
1981-1999 : Research Associate, 1st class, CNRS
1999- Director of Research, CNRS
2005- Director of the Laboratory of Parasite Vaccinology, Pasteur Institute

Research laboratoires

1969-1970 : Molecular Biology Institute, University of California, Los Angeles, U.S.A.
Director : Paul Boyer
1970-1976 : Department of Biochemistry, University of California, San Francisco, U.S.A.
Director : William J. Rutter
1976-1979 : Département de Microbiologie, Université de Genève
Directeur : Bernard Mach
1979-1984 : Unité d'Immunoparasitologie, Institut Pasteur, Paris
Directeur : Harvey Eisen
1984-1987 : Unité d'Immunogénétique Cellulaire, Institut Pasteur, Paris
Directeur : Jacques Thèze
1989-1995 : Unité d'Immunoparasitologie, Institut Pasteur, Paris
Directeur : Michel Rabinovitch; Luiz Pereira da Silva
1995-1998 : Unité de Parasitologie Expérimentale, Institut Pasteur, Paris
Directeur : Luiz Pereira da Silva
1998-1999 : Unité de Biologie des Interactions Hôte-Parasite, Institut Pasteur, Paris
Directeur : Catherine Breton
1999-2004 : Unité d'Immunologie Moléculaire des Parasites, Institut Pasteur, Paris
Directeur : Odile Puijalon
2005- Laboratoire de Vaccinologie Parasitaire, Institut Pasteur
Directeur: Shirley Longacre

Current scientific research interests

- Malaria: *Plasmodium falciparum* and *Plasmodium vivax*.
- Merozoite surface proteins (MSPs).
- Analysis of polymorphism of PfMSP1p42, PfMSP4 et PfMSP5 in endemic regions.
- Natural human immune responses to PfMSP1p19, PfMSP4 et PfMSP5 in endemic regions; associations with protective immunity.
- Development of malaria vaccines with efficiacy against both *P. falciparum* and *P. vivax* based on MSP1p19 : first clinical trial in 2007.
- Evaluation of new adjuvants and delivery systems for human vaccines.
- Development of a novel general approach to immunostimulation of subunit vaccines using recombinant antigens modified with glycosyl-phosphatidyl-inositol (GPI) moieties.

Publications of S. Longacre relating to MSP vaccines

Del Portillo, H.A., Longacre, S., Khouri, E. and David, P.H., 1991
Primary structure of the merozoïte surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species, **Proc. Natl. Acad. Scl., USA 88**, 4030-4034.

Snewin, V.A., Longacre, S. and David, P.H., 1991
Plasmodium vivax: older and wiser? **Res. Immunol. 142**, 631-635.

Longacre, S., Mendis, K. and David, P.H., 1994
Plasmodium vivax merozoïte surface protein 1 C-terminal recombinant proteins in baculovirus, **Mol. Biochem. Parasitol., 64**, 191-205.

Longacre, S., 1995
The *Plasmodium cynomolgi* merozoite surface protein 1 C-terminal sequence and its homologies with other *Plasmodium* species, **Mol. Biochem. Parasitol. 74**, 105- 111.

Holm, I., Nato, F., Mendis, K.N. and Longacre, S., 1997
Characterization of C-terminal merozoite surface protein-1 baculovirus recombinant proteins from *Plasmodium vivax* and *Plasmodium cynomolgi* as recognized by the natural anti-parasite immune response, **Mol. Biochem. Parasitol. 89**, 313-319.

Demangel, C., Rouyre, S., Alzari, P., Nato, F., Longacre, S. Lafaye, P. and Mazié, J-C, 1998
Phage-displayed mimotopes elicit monoclonal antibodies specific for a malaria vaccine candidate, **Biological Chemistry 379**, 65-70.

Perera, L., Handunnetti, S., Holm, I., Longacre, S. and Mendis, K., 1998
Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human *P. vivax* malaria, **Infect. Immun. 66,** 1500-1506.

Garraud, O., Diouf, A., Nguer, C.M., Dièye, A., Longacre, S., Kaslow, D., Holder, A., Tall, A., Molez, J.F., Perraut, R. and Mercereau-Puijalon, O., 1999
Different *Plasmodium falciparum* recombinant MSP1 19 antigens differ in their capacities to stimulate *in vitro* peripheral blood T lymphocytes in individuals from various endemic areas. **Scand. J. Immunol. 49 (4),** 431-440.

Garraud, O., Diouf, A., Holm, I., Nguer, C.M., Spiegel, A., Perraut, R. and Longacre, S., 1999
Secretion of parasite specific IgG by purified blood B lymphocytes from immune individuals after *in vitro* stimulation with recombinant *Plasmodium falciparum* merozoite surface protein-1 (19) antigen **Immunology** 97, 204-210.

Garraud, O., Diouf, A., Holm, I., Perraut, R. and Longacre, S., 1999
Immune responses to *P. falciparum*-merozoite surface protein 1 (MSP1) antigen: Induction of parasite specific immunoglobulin G in unsensitized human B cells after *in vitro* T cell priming with $MSP1_{19}$, **Immunology** 97, 497-505.

Chitarra, V., Holm, I., Bentley, G.A., Pêtres, and S., Longacre, S., 1999
The crystal structure of C-terminal merozoite surface protein 1 at 1.8 Å resolution, a highly protective malaria vaccine candidate, **Molecular Cell. 3,** 457-464.

Seneviratna, G.D., Manamperi, A.A., Kapilananda, G.M., Longacre, S., Handunnetti, S.M., and Udagama-Randeniya, P.V., 2000
A double antibody sandwich ELISA for the diagnosis of vivax malaria: a tool for further research, **Ceylon Journal of Medical Science 43,** 11-17.

Garraud, O., Perrault, R., Diouf, A., Nambel, W.S., Tall, A., Spiegel, A., Longacre, S., Kaslow, D.C., Jouin, H., Mattei, D., Engler, G.M., Nutman, T.B., Riley, E.M., Mercereau-Puijalon, O., 2002
Regulation of antigen-specific IgG subclasses to conserved and polymorphic *Plasmodium falciparum* antigens: An in vitro model. **Infect. Immun. 70,** 2820-2827.

Pizzaro, J.C., Chitarra, V., Verger, D., Holm, I., Pêtres, S., Dartevelle, S., Nato, F., Longacre, S. and Bentley, G. A., 2003
Crystal structure of a Fab complex formed with PfMSP1-19, the C-terminal fragment of merozoite surface protein 1 from *Plasmodium falciparum* : a malaria vaccine candidate. **J. Mol. Biol.** 328, 1091-1103.

Perraut R., Marrama L., Diouf B., Sokna C., Tall A., Nabeth P., Trape J.-F., Longacre S., & Mercereau-Puijalon O., 2004
Antibodies to the conserved C-terminal domain of the *Plasmodium falciparum* merozoite surface protein-1 and to the merozoite extract and their relationship with *in vitro* inhibitory antibodies and protection against clinical malaria in a Senegalese village. **J. Infect. Dis.** 191, 264-271.

Jouin, H., Garraud, O., Longacre, S., Baleux, F., Mercereau-Puijalon, O. and Milon, G., 2005
Human antibodies to the polymorphic block 2 domain of the *Plasmodium falciparum* Merozoite Surface Protein 1 (MSP-1) exhibit a highly skewed, peptide-specific light chain distribution. **Immunology and Cell Biology** 83,392-395.

Perraut, R., Diatta, B., Marrama, L., Garraud, O., Jambou, R., Longacre, S., Krishnegowda, G., Dieye, A., Gowda, C., 2005
Differential Antibody Responses to *P. falciparum* Glycosylphosphatidylinositol Anchors in Patients With Cerebral and Mild Malaria. **Microbes and Infection** 7, 682-687.

Polson, H., Conway, D., Fandeur, T., Mercereau-Puijalon, O., Longacre, S., 2005
Gene polymorphism of *Plasmodium falciparum* merozoite surface proteins 4 and 5, **Mol. Biol. Parasit.** 142, 110-115.

Christophe Tournamille[1], Anne Filipe[1], Cyril Badaut[2], Shirley Longacre[3], Jean-Pierre Cartron[1], Caroline Le Van Kim[1] and Yves Colin[1], 2005
Fine mapping of the interaction site of the Duffy Antigen/Receptor for Chemokines to the *Plasmodium vivax* Duffy-binding protein. **Mol. Biol. Parasit.** 144, 100-103.

Bonnet, S., Pêtres, S., Holm, I., Fontaine, T., Rosario, S., Roth, C., and Longacre, S., 2006

Soluble and glyco-lipid modified baculovirus *Plasmodium falciparum* C-terminal Merozoite Surface Protein 1, two forms of a leading malaria vaccine candidate. **Vaccine,** in press.

Annex II

INFECTION AND IMMUNITY, Sept. 1996, p. 3614–3619 Vol. 64, No. 9
0019-9567/96/$04.00+0

Immunization of *Aotus nancymai* with Recombinant C Terminus of *Plasmodium falciparum* Merozoite Surface Protein 1 in Liposomes and Alum Adjuvant Does Not Induce Protection against a Challenge Infection

PETRA A. BURGHAUS,[1]† BRUCE T. WELLDE,[2] TED HALL,[2] ROBERTA L. RICHARDS,[3] ANDREA F. EGAN,[4] ELEANOR M. RILEY,[4] W. RIPLEY BALLOU,[2] AND ANTHONY A. HOLDER[1]*

Division of Parasitology, National Institute for Medical Research, London,[1] and Division of Biological Sciences, Institute for Cell, Animal and Population Biology, University of Edinburgh, Edinburgh,[4] United Kingdom, and Departments of Immunology[2] and Membrane Biochemistry,[3] Walter Reed Army Institute for Research, Washington, D.C.

Received 26 February 1996/Returned for modification 12 April 1996/Accepted 1 July 1996

Merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum* is an antimalarial vaccine candidate. The highly conserved 19-kDa C-terminal processing fragment of MSP-1 ($MSP\text{-}1_{19}$) is of particular interest since it contains epitopes recognized by monoclonal antibodies which inhibit the invasion of erythrocytes in vitro. The presence of naturally acquired anti-$MSP\text{-}1_{19}$ antibodies in individuals exposed to malaria has been correlated with reduced morbidity, and immunization with an equivalent recombinant *P. yoelii* antigen induces substantial protection against this parasite in mice. We have expressed *P. falciparum* $MSP\text{-}1_{19}$ in *Escherichia coli* as a correctly folded protein and immunized *Aotus nancymai* monkeys by using the protein incorporated into liposomes and adsorbed to alum. After vaccination, the sera from these animals contained anti-$MSP\text{-}1_{19}$ antibodies, some of which competed for binding to $MSP\text{-}1_{19}$ with monoclonal antibodies that inhibit parasite invasion of erythrocytes in vitro. However, after challenge with either a homologous or a heterologous strain of parasite, all animals became parasitemic and required treatment. The immunization did not induce protection in this animal model.

Malaria is a serious health problem in tropical countries, with an estimated 300 to 500 million clinical cases and 2.7 million deaths per year (9). Resistance to existing drugs is developing fast, and an effective vaccine is urgently needed. A number of antigens expressed at different stages of the parasite's life cycle have been characterized with respect to their use in a subunit vaccine against *Plasmodium falciparum* (19). Merozoite surface protein 1 (MSP-1) is one of the most promising vaccine candidates (14, 20). People naturally exposed to *P. falciparum* develop antibodies against MSP-1 (15, 27, 31–33). Furthermore, an association between a naturally acquired immune response to MSP-1 and reduced malaria morbidity has been observed (30). In a number of independent studies, immunization with purified native MSP-1 or recombinant fragments of the protein has induced at least partial protection against parasite challenge (reviewed in reference 14).

MSP-1 is one of the best-characterized *P. falciparum* proteins (reviewed in reference 10). At the time of merozoite release and erythrocyte invasion, MSP-1 is proteolytically cleaved into several fragments. Only a 19-kDa C-terminal polypeptide ($MSP\text{-}1_{19}$) is carried into newly infected erythrocytes, and the remaining fragments are shed from the parasite surface (3). The sequence of $MSP\text{-}1_{19}$ is highly conserved and composed of two motifs which have structural similarity to epidermal growth factor (4). Epitopes in this region of MSP-1 are the targets of antibodies which inhibit erythrocyte invasion in vitro (3, 7, 11, 29). Vaccination experiments with the equivalent polypeptide from *P. yoelii* expressed in *Escherichia coli* have shown that mice immunized with this recombinant protein are protected against an otherwise lethal challenge with this rodent parasite (12, 25). Protection appears to be mediated largely by antibody (16, 25). *P. falciparum* $MSP\text{-}1_{19}$ expressed in *E. coli* or *Saccharomyces cerevisiae* appears to be correctly folded (6, 23) and reacts with antibodies induced by natural infection (15, 33). In The Gambia, where malaria is endemic, the prevalence of anti-$MSP\text{-}1_{19}$ antibodies is low in children (about 20%) but is up to 60% in adults (15), and a significant correlation between anti-$MSP\text{-}1_{19}$ antibody titer and reduced malaria morbidity has been reported (16). Acquisition of high levels of antibodies to $MSP\text{-}1_{19}$ from the mother correlates with a reduced probability of an episode of clinical malaria in infants (18). In summary, in vitro studies, results obtained with a rodent malaria model, and studies on naturally acquired immune responses suggest that $MSP\text{-}1_{19}$ is an important target of antimalarial immunity and for the development of a vaccine against *P. falciparum*.

To evaluate the recombinant *P. falciparum* $MSP\text{-}1_{19}$ expressed in bacteria as a vaccine against malaria, we immunized *Aotus nancymai* monkeys and challenged them with blood stage parasites.

MATERIALS AND METHODS

Production of antigen. The antigen used in this study was the C terminus of the Wellcome type MSP-1 expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST). The protein was prepared as described previously (6). The sequence contains amino acids Asn-1631 to Asn-1726 (numbering according to reference 26). To purify the malaria polypeptide for use in enzyme-linked immunosorbent assays (ELISA), GST–$MSP\text{-}1_{19}$ was cleaved with protease factor

* Corresponding author. Mailing address: Division of Parasitology, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. Phone: (44) 181 959 3666, ext. 2175. Fax: (44) 181 913 8593. Electronic mail address: a-holder@nimr.mrc.ac.uk.

† Present address: ZMBH, Universität Heidelberg, D-69120 Heidelberg, Germany.

Xa (Boehringer) and MSP-1_{19} was separated from GST by gel filtration through Superdex-75 (Pharmacia).

Formulation of vaccine. GST–MSP-1_{19} and GST were formulated with liposomes that were prepared as described previously (1). The liposomes were composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol in a molar ratio of 9:1:7.5 and lipid A at 13 μg/mol of phospholipid. The phospholipids were obtained from Avanti Polar-Lipids, Inc., Alabaster, Ala.; the lipid A (from *Salmonella minnesota* R595) was obtained from List Biological Laboratories, Inc., Campbell, Calif. A single dose of 250 μl of liposomes contained 215 μg of lipid A, 16.5 μmol of phospholipid, and 74 μg of the GST–MSP-1_{19} fusion protein or 40 μg of GST as a control antigen, respectively. The liposomes were adsorbed to alum prior to injection.

Design of vaccine trial. *A. nancymai* monkeys were immunized in two separate trials with two different *P. falciparum* challenge strains. For homologous challenge, parasites of the FVO strain of *P. falciparum* were used; these parasites express MSP-1_{19} with a sequence identical to that of the recombinant protein (23). The FVO strain is a highly virulent parasite for *A. nancymai*, producing a rapidly developing parasitemia after challenge. To preserve monkeys infected with this strain, treatment with mefloquine (20 mg/kg orally) was initiated when the percentage of infected erythrocytes approached 5%. Heterologous challenge was done with *P. falciparum* CAMP parasites, which express the MAD20 allele of the *msp-1* gene and the amino acid sequence of whose MSP-1_{19} differs from that of the antigen at four positions (26). The CAMP strain is less virulent, with only about 40% of infected *A. nancymai* monkeys requiring treatment (chloroquine-HCl, 5 mg intramuscularly on each of 3 days) for parasitemias approaching 10%. Twenty-five percent require treatment for severe anemia (<20% hematocrit) with moderate parasitemia, and approximately 35% survive without treatment, never developing high parasitemia or severe anemia, but nontheless undergo substantial infections (2, 34). Eleven animals were used: numbers 1, 2, 3, 7, and 8 were immunized with GST, and numbers 4, 5, 6, 9, 10, and 11 were immunized with GST–MSP-1_{19}. Monkeys 1 to 6 were challenged with the CAMP strain, and numbers 7 to 11 were challenged with the FVO strain.

Immunization and challenge protocol. The monkeys were immunized by the intramuscular route on days 1, 30, and 60 and challenged on day 67 with 10^5 parasitized erythrocytes obtained from donor monkeys. Blood samples were taken immediately before each immunization and on the day of challenge. After challenge, parasitemia was monitored with Giemsa-stained blood smears and hematocrits were determined daily. Monkeys were treated with antimalarial drugs when the parasitemia approached 10% for the CAMP strain and 5% for the more virulent FVO strain or when the hematocrit dropped to 20% or less.

ELISA. Immunolon 4 microtiter plates (Dynatech Laboratories) were coated by incubation with 3 μg of MSP-1_{19} per ml or 2 μg of GST per ml in carbonate buffer (pH 9.5) at 5°C and washed three times with phosphate-buffered saline, and then uncoated sites were blocked with 1% (wt/vol) milk powder in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20. The plates were washed again, and then triplicate or duplicate wells were incubated with serially diluted sera. After washing, alkaline phosphatase-conjugated anti-monkey immunoglobulin G (Sigma) was added at a dilution of 1/1,000. Bound antibody was detected with *p*-nitrophenylphosphate (Sigma), and the A_{405} was measured.

Competition ELISA. The ability of antibody present in the monkey sera to block binding to MSP-1_{19} of monoclonal antibodies (MAbs) 2.2, 7.5, 12.8, and 12.10 (a kind gift of J. S. McBride) was studied. Two of these MAbs, 12.8 and 12.10, block parasite invasion of erythrocytes in vitro (11) and inhibit the proteolytic cleavage of MSP-1 (5), whereas the others do not. Plates coated with MSP-1_{19} were incubated with various dilutions of monkey sera and incubated for 5 h. After the plates were washed, ascitic fluid containing one MAb and diluted 1/4,000 was added and the plates were incubated overnight. Finally, the plates were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G diluted 1/1,000. Bound antibody was detected by using H_2O_2 and *o*-phenylenediamine (Sigma); the color reaction was stopped by addition of H_2SO_4, and then the A_{492} was measured.

RESULTS

Assessment of antibody response by ELISA. All of the animals tolerated the immunizations well, with no deleterious effects on weight or hematologic parameters. The sera collected after immunization and immediately before challenge were analyzed for the presence of specific antibodies. We determined the titers of antibodies specific for GST and MSP-1_{19}.

The antigen used for immunization was either the GST–MSP-1_{19} fusion protein or GST alone. Therefore, to assess the immunogenicity of the vaccine formulation, we determined the response in each animal to GST by ELISA. As shown in Fig. 1a and b, all of the animals developed antibodies against GST, indicating that the vaccine formulation had stimulated an immune response. The response tended to be lower in animals that had received the fusion protein, and monkey 11 developed a noticeably lower titer than all of the others.

We also investigated the specific antibody response to MSP-1_{19} by using the cleaved and purified product as the antigen in the ELISA. All of the monkeys that had been immunized with GST–MSP-1_{19} developed antibodies against this protein (Fig. 1c and d), whereas the animals immunized with GST did not. Monkey 11 also developed a lower response to GST–MSP-1_{19} than did the others immunized with this antigen.

Antibodies induced by immunization compete with MAbs for antigen binding. The sera were assayed for the ability to block the binding to recombinant protein of inhibitory MAbs 12.8 and 12.10, which recognize different epitopes. The sera of monkeys immunized with GST–MSP-1_{19} had at least a partial effect on the binding of both MAbs at the dilutions tested (Fig. 2), whereas the sera from the GST-vaccinated animals had no effect on MAb binding. In competition with MAb 12.8 (Fig. 2a and b), blocking was first observed with some samples at a dilution of 1/250 and was essentially complete at a dilution of 1/25, the highest concentration in serum tested. In contrast, the blocking of MAb 12.10 binding was first detected at a concentration in serum of 1/50 and at the highest concentration tested (1/25), the binding of MAb 12.10 was blocked to only 60% by four of the sera and to 45 and 35% by two others (Fig. 2c and d). The strongest blocking effect was observed for noninhibitory MAb 2.2. In this case, competition was first detected at a dilution of 1/5,000 in some samples (Fig. 2e and f).

Outcome of challenge with parasites. All six monkeys (numbers 1 to 6) in the control (GST) and immunized (GST–MSP-1_{19}) groups challenged with the heterologous strain (CAMP) developed detectable parasitemia in their blood on day 3 or 4 after challenge (Fig. 3a and b). While one control monkey required treatment for high parasitemia on day 15, the other two controls both recovered without treatment after undergoing substantial infections. Two of the immunized monkeys required treatment for high parasitemia on days 10 and 11 after challenge. The third immunized monkey developed moderate parasitemia but was treated because of severe anemia on day 13.

The outcome of challenge with the homologous strain (FVO) is shown in Fig. 3c and d (numbers 7 to 11). Parasites were detected in the blood of all six monkeys on day 4 or 5 after challenge, and all monkeys were treated on day 10 because of high parasitemia. While the two specific control (GST) animals had somewhat higher parasitemias than the three immunized monkeys at the time of treatment, two other control monkeys challenged at the same time with the same inoculum had parasitemias on day 10 of 195,950 and 664,620 organisms per μl of blood, respectively (data not shown).

DISCUSSION

Immunization with the C terminus of *P. yoelii* MSP-1 expressed in bacteria as a fusion protein with GST can protect mice against an otherwise lethal challenge with this parasite (12, 25). In contrast, immunization of mice with *P. chabaudi* MSP-1_{19} expressed in *E. coli* as a fusion with maltose-binding protein was not effective (28). We have produced the homologous region of *P. falciparum* MSP-1 as a GST fusion protein and tested it as a vaccine in *A. nancymai* monkeys challenged with this human malaria parasite. In the protocol used here, vaccination with the C terminus of MSP-1 did not induce protection against a challenge with *P. falciparum*.

In the *P. yoelii* model, the secondary structure of the antigen is important for induction of protective immunity; for example, reduced and alkylated recombinant protein does not induce

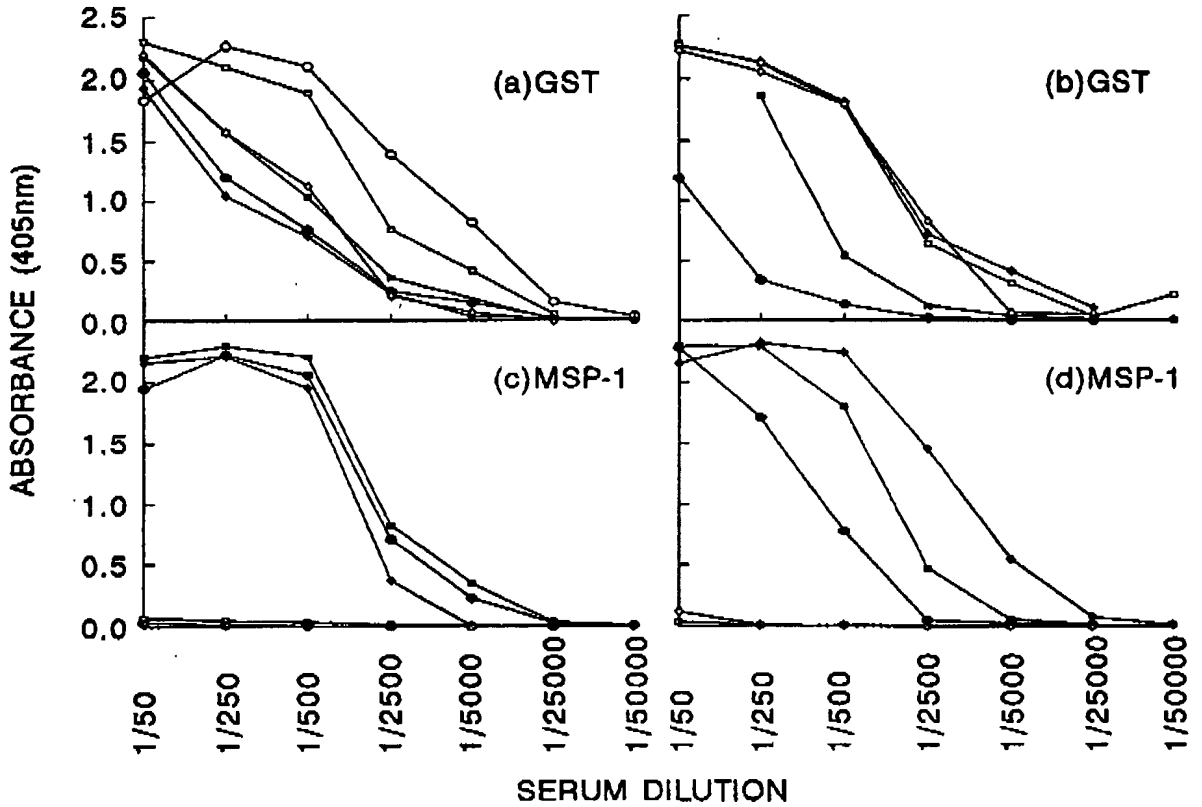

FIG. 1. Antibody titers of *Aotus* sera as determined by ELISA. The capture antigen was control protein GST (a and b) or malarial protein MSP-1_{19} (c and d). Monkeys were challenged with the heterologous parasite strain (no. 1 to 6) (a and c) or given a homologous challenge (no. 7 to 11) (b and d). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST–MSP-1_{19}. Monkey no. (symbol): 1 (◇), 2 (□), 3 (○), 4 (◆), 5 (■), 6 (●), 7 (◇), 8 (□), 9 (◆), 10 (■), and 11 (●).

protection. *P. falciparum* MSP-1_{19} contains 12 cysteine residues and it is thought that the six predicted disulfide bonds are important to determine the three-dimensional structure of the polypeptide. The recombinant protein produced in *E. coli* has been shown to contain epitopes shared with parasite-derived MSP-1 by using a panel of 12 MAbs and several immune sera (6, 15, 16, and unpublished data). Five of the MAbs recognize epitopes in the first epidermal growth factor-like motif (8), two (MAbs 91.33.5 and 94.65, a kind gift of G. S. N. Hui) are specific for the second epidermal growth factor-like motif (23), and the rest bind only when both motifs are expressed together. None of the MAbs binds when the protein has been treated with reducing and alkylating agents. These studies suggest that the recombinant protein is correctly folded and resembles the native protein.

Immunization with GST–MSP-1_{19} induced the production of antibodies, some of which may be against functionally important epitopes, but the antibody titers were low compared with those in mice protected against challenge infection. In mice immunized with recombinant *P. yoelii* MSP-1_{19}, protection appears to correlate with the antibody response (12, 13, 25). In this study, the monkeys responded to immunization with the fusion protein. They developed antibodies to both the GST carrier and MSP-1_{19} (Fig. 1); only one monkey (number 11) did not respond well on immunization. The sera also partially blocked the binding to MSP-1_{19} of MAbs that inhibit erythrocyte invasion in vitro (Fig. 2). These MAbs have different specificities; 12.8 recognizes an epitope in the first epidermal growth factor-like motif, whereas MAb 12.10 binds only to the whole MSP-1_{19} polypeptide and not to either of the individual epidermal growth factor-like motifs alone (6, 8). The results of this competition ELISA indicate that the animals developed at least some antibodies recognizing epitopes that are important targets for inhibition of the invasion process, as judged by in vitro assays. However, these antibodies were present in a low concentration because high concentrations of serum were required in the blocking assay, in particular with MAb 12.10. The majority of antibodies induced by immunization may be directed against other epitopes of MSP-1_{19}. Consistent with this observation is the fact that the sera were able to effectively block the binding of noninhibitory MAb 2.2 and that of MAb 7.5 to a lesser extent (data not shown). Antibodies with 2.2 and 7.5 specificity compete with the inhibitory antibodies for binding and prevent the inhibition of MSP-1 processing (5) and therefore may be detrimental to protection.

After challenge, none of the monkeys that received the

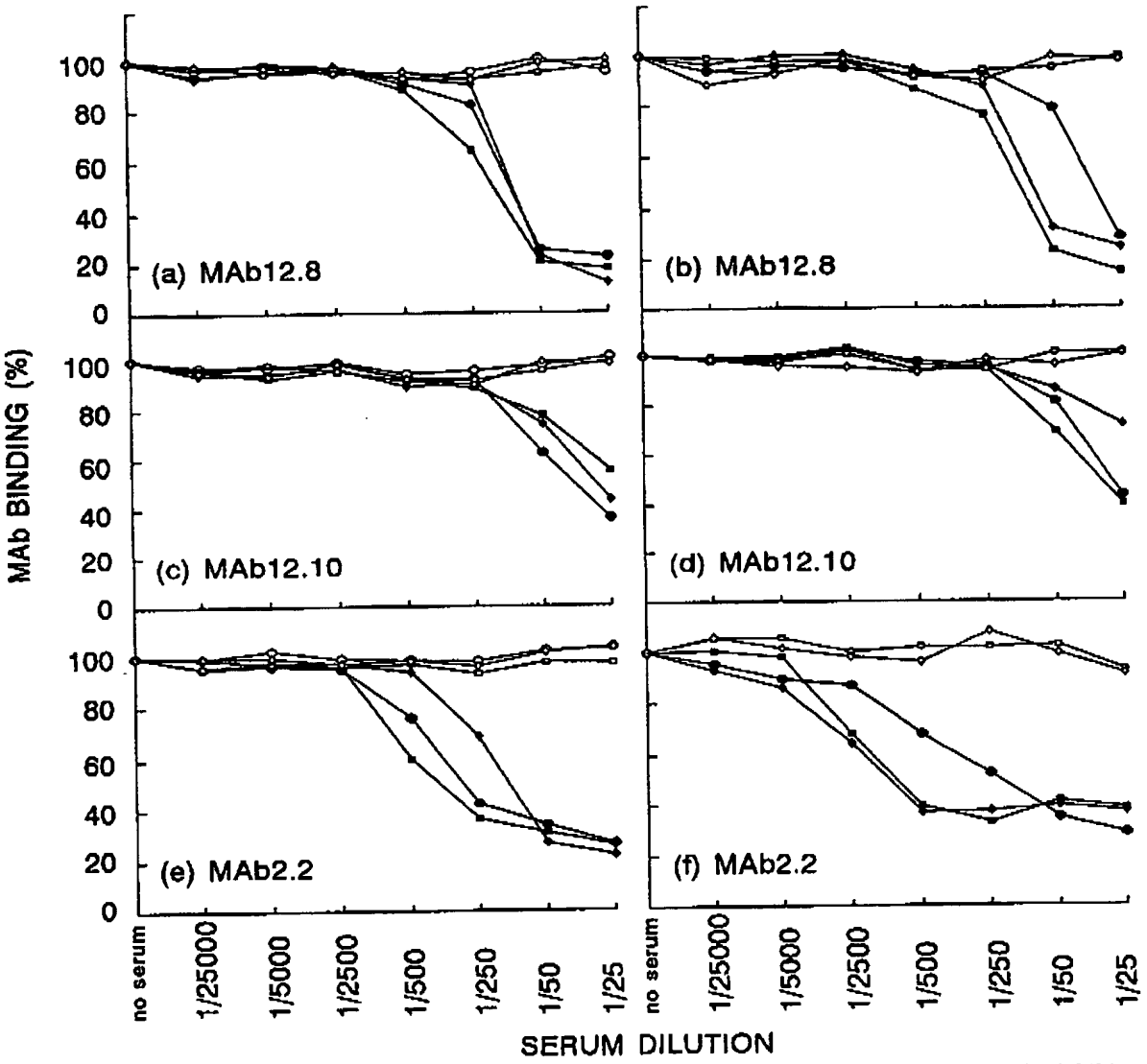

FIG. 2. Competition ELISA to demonstrate that the monkey sera contained antibodies that block the binding of inhibitory MAbs 12.8 (a and b) and 12.10 (c and d) and noninhibitory MAb 2.2 (e and f). Monkeys were given a heterologous challenge (a, c, and e) or a homologous challenge (b, d, and f). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST-MSP-1_{19}. Monkey no. (symbol): 1 (◇), 2 (□), 3 (○), 4 (◆), 5 (■), 6 (●), 7 (◇), 8 (□), 9 (◆), 10 (■), and 11 (●).

GST–MSP-1_{19} vaccine was protected and all required chemotherapy, although they had developed antibodies against the antigen. This contrasts with immunity induced by infection and cure in *A. nancymai*, which is antibody dependent and of long duration (34). Recently, Kumar and colleagues used *P. falciparum* MSP-1_{19} expressed in *S. cerevisiae* (23) and formulated with Freund's adjuvant (24) to immunize *Aotus* monkeys of two different karyotypes. The two *A. nancymai* monkeys which received the MSP-1_{19} vaccine had peak parasitemias of <0.1% and resolved the infection. It was suggested that the protective immunity induced was not mediated by antibodies that block invasion. A significant difference between the two studies is the adjuvant used. Freund's adjuvant is unacceptable for human use, and therefore we used liposomes and alum, an adjuvant combination which is being developed for use in humans (17). In the *P. yoelii* model, essentially the same GST–MSP-1_{19}–liposome formulation did induce protection in mice (24a). For purified MSP-1 (21) and MSP-1_{19} (22), the importance of an adjuvant for the level and quality of the immune response in different species has been demonstrated.

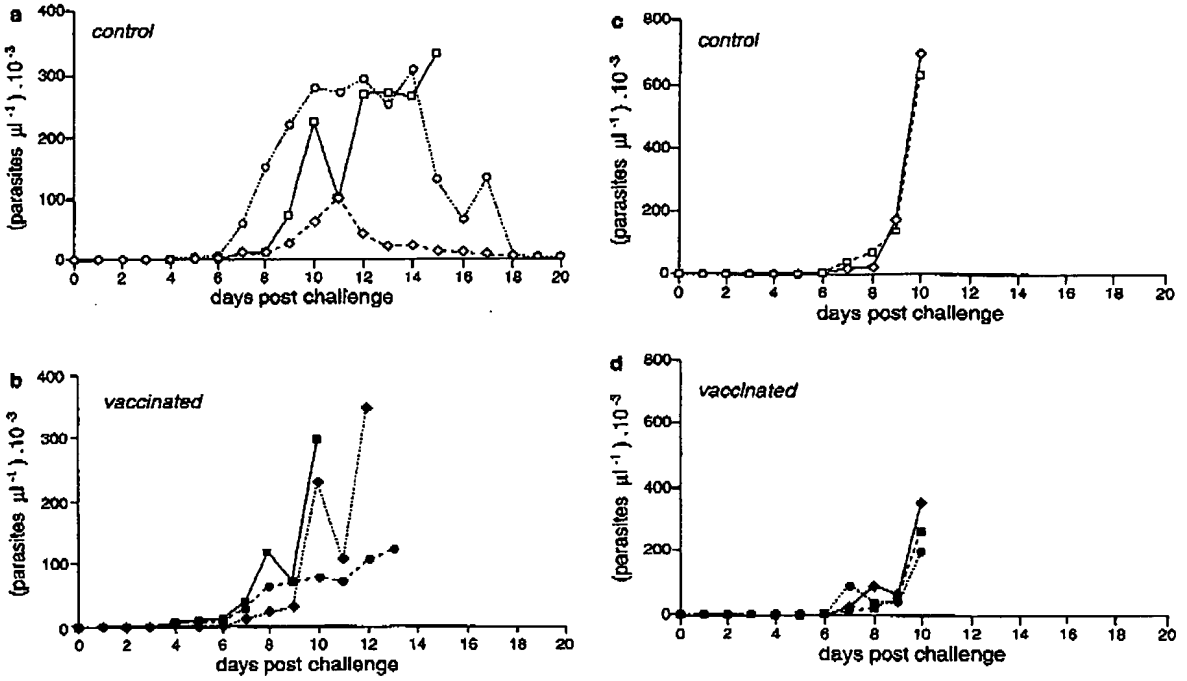

FIG. 3. Parasitemia curves of *Aotus* monkeys challenged with the heterologous CAMP strain (a and b) or the homologous FVO strain (c and d). Open symbols represent control monkeys immunized with GST; filled symbols represent monkeys immunized with GST–MSP-1_{19}. Monkey no. (symbol): 1 (◇), 2 (□), 3 (○), 4 (◆), 5 (■), 6 (●), 7 (◇), 8 (□), 9 (◆), 10 (■), and 11 (●).

The vaccination of a small group of *Aotus* monkeys in this study resulted in an immune response, but there was no apparent influence on the course of the disease. When the results of in vitro assays (3, 7), observations in field studies with naturally exposed humans (16, 18, 33), results of immunization studies with two mouse models (12, 25, 28), and results obtained with *Aotus* monkeys given a strong adjuvant (24) or liposomes-alum (this work) are combined, there is still no clear picture of the importance of MSP-1_{19} in immunity to blood stage *P. falciparum* malaria. The in vitro assays and results obtained with *P. yoelii* suggest that high concentrations of antibody to MSP-1_{19} can prevent erythrocyte invasion. It remains to be determined whether or not the antibody concentration, the fine specificity of the humoral response (5), or the contribution of an unidentified cellular response is crucial for protection in vivo. The design of a vaccine based on the sequence of MSP-1 to elicit the production of specific inhibitory antibodies in humans remains to be implemented and requires the further development of reliable systems to test immunogenicity and efficacy in vitro or in animal models.

ACKNOWLEDGMENTS

We thank Jana S. McBride and George S. N. Hui for the gift of antibodies.

This work received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. **Alving, C. R., S. Shichijo, I. Mattsby-Baltzer, R. L. Richards, and N. M. Wassef.** 1993. Preparation and use of liposomes in immunological studies, p. 317–343. *In* G. Gregoriadis (ed.), Liposome technology, 2nd ed., vol. 3. CRC Press, Boca Raton, Fla.
2. **Berzins, K., H. Perlmann, B. Wahlin, H.-P. Ekre, B. Högh, E. Petersen, B. Wellde, M. Schoenbechler, J. Williams, J. Chulay, and P. Perlmann.** 1991. Passive immunization of *Aotus* monkeys with human antibodies to the *Plasmodium falciparum* antigen Pf155/RESA. Infect. Immun. 59:1500–1506.
3. **Blackman, M. J., H.-G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. 172:379–382.
4. **Blackman, M. J., I. T. Ling, S. C. Nicholls, and A. A. Holder.** 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. Mol. Biochem. Parasitol. 49:29–34.
5. **Blackman, M. J., T. J. Scott-Finnigan, S. Shai, and A. A. Holder.** 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J. Exp. Med. 180:389–390.
6. **Burghaus, P. A., and A. A. Holder.** 1994. Expression of the 19-kilodalton carboxy-terminal fragment of the *Plasmodium falciparum* merozoite surface protein-1 in *Escherichia coli* as a correctly folded protein. Mol. Biochem. Parasitol. 64:165–169.
7. **Chang, S. P., H. L. Gibson, C. T. Lee-Ng, P. J. Barr, and G. S. N. Hui.** 1992. A carboxyl-terminal fragment of *Plasmodium falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth. J. Immunol. 149:548–555.
8. **Chappel, J. A., and A. A. Holder.** 1993. Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion in vitro recognize the first growth factor-like domain of merozoite surface protein-1. Mol. Biochem. Parasitol. 60:303–312.
9. **Cohen, J.** 1994. Frontiers in medicine: vaccines—bumps on the vaccine road. Science 265:1371–1372.
10. **Cooper, J. A.** 1993. Merozoite surface antigen-1 of *Plasmodium*. Parasitol. Today 9:50–54.
11. **Cooper, J. A., I. T. Cooper, and A. J. Saul.** 1992. Mapping of the region predominantly recognized by antibodies to the *Plasmodium falciparum* merozoite surface antigen MSA-1. Mol. Biochem. Parasitol. 51:301–312.
12. **Daly, T. M., and C. A. Long.** 1993. A recombinant 15-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii yoelii* 17XL merozoite surface pro-

tein 1 induces a protective immune response in mice. Infect. Immun. 61: 2462–2467.
13. **Daly, T. M., and C. A. Long.** 1995. Humoral response to a carboxyl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria. J. Immunol. 155:236–243.
14. **Diggs, C. L., W. R. Ballou, and L. H. Miller.** 1993. The major merozoite surface protein as a malaria vaccine target. Parasitol. Today 9:300–302.
15. **Egan, A. F., J. A. Chappel, P. A. Burghaus, J. S. Morris, J. S. McBride, A. A. Holder, D. C. Kaslow, and E. M. Riley.** 1995. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1-19, the carboxy-terminal fragment of the major merozoite surface protein of *Plasmodium falciparum*. Infect. Immun. 63:456–466.
16. **Egan, A. F., J. Morris, G. Barnish, S. Allen, B. M. Greenwood, D. C. Kaslow, A. A. Holder, and E. M. Riley.** 1996.Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. J. Infect. Dis. 173:765–769.
17. **Fries, L. F., D. M. Gordon, R. L. Richards, J. E. Egan, M. R. Hollingdale, M. Gross, C. Silverman, and C. R. Alving.** 1992. Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. USA 89:358–362.
18. **Hogh, B., N. T. Marbiah, P. A. Burghaus, and P. K. Andersen.** 1995. Relationship between maternally derived anti-*Plasmodium falciparum* antibodies and risk of infection and disease in infants living in an area of Liberia, West Africa, in which malaria is highly endemic. Infect. Immun. 63:4034–4038.
19. **Holder, A. A.** 1993. Developments with anti-malaria vaccines. Ann. N. Y. Acad. Sci. 700:7–21.
20. **Howard, R. J., and B. L. Pasloske.** 1993. Target antigens for asexual malaria vaccine development. Parasitol. Today 9:369–374.
21. **Hui, G. S. N., and S. P. Chang.** 1992. *Plasmodium falciparum*: induction of biologically active antibodies to gp195 is dependent on the choice of adjuvants. Exp. Parasitol. 75:155–157.
22. **Hui, G. S. N., W. L. Gosnell, S. E. Case, C. Hashiro, C. Nikaido, A. Hashimoto, and D. C. Kaslow.** 1994. Immunogenicity of the C-terminal 19-kDa fragment of the *Plasmodium falciparum* merozoite surface protein 1 (MSP1), $YMSP1_{19}$ expressed in *S. cerevisiae*. J. Immunol. 153:2544–2553.
23. **Kaslow, D. C., G. Hui, and S. Kumar.** 1994. Expression and antigenicity of *Plasmodium falciparum* major merozoite surface protein (MSP1-19) variants secreted from *Saccharomyces cerevisiae*. Mol. Biochem. Parasitol. 63:283–289.
24. **Kumar, S., A. Yadava, D. B. Keister, J. H. Tian, M. Ohl, K. A. Perdue-Greenfield, L. H. Miller, and D. C. Kaslow.** 1995. Immunogenicity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in Aotus monkeys. Mol. Med. 1:1–8.
24a. **Ling, I. T., et al.** Unpublished data.
25. **Ling, I. T., S. A. Ogun, and A. A. Holder.** 1994. Immunisation against malaria with a recombinant protein. Parasite Immunol. 16:63–67.
26. **Miller, L. H., T. Roberts, M. Shahabuddin, and T. F. McCutchan.** 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP1). Mol. Biochem. Parasitol. 59:1–14.
27. **Müller, H.-M., K. Früh, A. von Brunn, F. Esposito, S. Lombardi, A. Crisanti, and H. Bujard.** 1989. Development of the human immune response against the major merozoite surface protein (gp190) of *Plasmodium falciparum*. Infect. Immun. 57:3765–3769.
28. **O'Dea, K. P., P. G. McKean, W. Jarra, and K. N. Brown.** 1996. A single copy merozoite antigen and immune evasion. Parasite Immunol. 18:165–172.
29. **Pirson, P. J., and M. E. Perkins.** 1985. Characterization with monoclonal antibodies of a surface antigen of *Plasmodium falciparum* merozoites. J. Immunol. 134:1946–1951.
30. **Riley, E. M., S. J. Allen, J. G. Wheeler, M. J. Blackman, S. Bennett, B. Takacs, H. J. Schoenfeld, A. A. Holder, and B. M. Greenwood.** 1992. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. Parasite Immunol. 14:321–337.
31. **Riley, E. M., S. Morris-Jones, M. J. Blackman, B. M. Greenwood, and A. A. Holder.** 1993. A longitudinal study of naturally acquired cellular and humoral immune responses to a merozoite surface protein (MSP1) of *Plasmodium falciparum* in an area of seasonal transmission. Parasite Immunol. 15:513–524.
32. **Tolle, R., K. Früh, O. Doumbo, O. Koita, M. N'Diaye, A. Fischer, K. Dietz, and H. Bujard.** 1993. A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections. Infect. Immun. 61:40–47.
33. **Udhayakumar, V., D. Anyona, S. Kariuki, Y. P. Shi, P. B. Bloland, O. H. Brauch, W. Weiss, B. L. Nahlen, D. C. Kaslow, and A. A. Lal.** 1995. Identification of T and B cell epitopes recognized by humans in the C-terminal 42-kDa domain of the *Plasmodium falciparum* merozoite surface protein (MSP)-1. J. Immunol. 154:6022–6030.
34. **Wellde, B. T., and T. Hall.** Unpublished data.

Editor: S. H. E. Kaufmann